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Heterotrimeric G protein α and β subunits antagonistically modulate stomatal density in *Arabidopsis thaliana*

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ABSTRACT

Stomata are essential for efficient gas and water-vapor exchange between the atmosphere and plants. Stomatal density and movement are controlled by a series of signal molecules including phytohormones and peptides as well as by environmental stimuli. It is known that heterotrimeric G-proteins play an important role in the ABA-inhibited stomatal opening. In this study, the G-protein signaling pathway was also found to regulate stomatal density on the lower epidermis of *Arabidopsis* cotyledons. The loss-of-function mutation of the G-protein α-subunit (*GPA1*) showed a reduction in stomatal density, while overexpression of the constitutively active form of $GPA1^{QL}$ increased stomatal density, indicating a positive role of the active form of GPA1 in stomatal development. In contrast, stomatal density increased in the null mutant of the G-protein β-subunit (*AGB1*) but decreased in transgenic lines that overexpressed *AGB1*. Stomatal analysis of the *gpa1 agb1* double mutants displayed an average value of stomatal density compared to the single mutants. Taken together, these results suggest that the stomatal density in *Arabidopsis* is modulated by GPA1 and AGB1 in an antagonistic manner.

Introduction

The stoma, a small pore surrounded by a pair of guard cells, controls exchanges of gas and water-vapor between plants and atmosphere, and thus is critical for photosynthesis and water use efficiency (Hetherington and Woodward, 2003). Stomatal development is characterized by a series of epidermal cell divisions in *Arabidopsis* (Nadeau and Sack, 2003). First, Undifferentiated epidermal cells, also called meristemoid mother cells (MMC), divide asymmetrically to produce a small cell, namely meristemoids, and a larger sister cell. This asymmetrical division is named the entry division to initiate the stomatal lineage. Second, meristemoids undergo up to several rounds of asymmetric divisions, namely amplifying divisions, either to increase the number of the total epidermal cells or to convert into guard mother cells (GMC). On the other hand, the larger sister cell can become a pavement cell or undergo spacing divisions, which prevent stomata from direct contact each other, to generate satellite meristemoids. Lastly, GMCs divide symmetrically to form a pair of guard cells. Therefore, stomatal number depends on the frequency of these three asymmetrical cell divisions of the larger sister cells and meristemoids (Bergmann and Sack, 2007).

Recently, a significant progress has been made towards identification of components in the pathway of the stomatal cell lineage. Genetic analyses reveal a main linear pathway initiated by leucine-rich repeat (LRR) receptor-like kinases (Bergmann and Sack, 2007). The subtilisin-like protease STOMATAL DENSITY and DISTRIBUTION 1 (SDD1) may generate a cell–cell signal that is recognized by a LRR receptor-like kinase, TOO MANY MOUTHS (TMM) together with other three ERECTA family receptor-like kinases, ERECTA, ERECTA-like 1 (ERL1) and ERL2 (Berger and Altmann, 2000; Von Groll et al., 2002; Nadeau and Sack, 2002). The signal is transmitted from receptors into the nucleus through a mitogen-activated protein kinase (MAPK) cascade, which is composed of YODA (a MAPKKK), MKK4/MKK5, and MPK3/MPK6 (Bergmann et al., 2004; Wang et al., 2007). Although the direct downstream effectors of this MAPK cascade are unclear, three critical transcription factors containing the basic helix-loop-helix domain have been demonstrated to regulate sequential steps in stomatal differentiation: SPEECHLESS (SPCH) commences the first asymmetric cell division to produce MMCs; MUTE is required for termination of the asymmetric division activity and promotion of differentiation from meristemoids to GMCs; and FAMA regulates the last step of stomatal development to promote guard cell differentiation (MacAlister et al., 2007; Pillitteri et al., 2007; Ohashi-Ito and Bergmann, 2006).

In addition to these developmental or genetic factors, stomatal density and distribution on the epidermal layer of cotyledons, stalks and leaves are also influenced by environmental cues (Hetherington and Woodward, 2003; Gray et al., 2000). Many environmental factors such as humidity (Schürmann, 1959), temperature (Srivastava et al., 1995), CO_2 partial pressure (Clifford et al., 1995; Gray et al., 2000), and light intensity (Rahim and Fordham, 1991) have been demonstrated to

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modulate stomatal density or stomatal index. In *Arabidopsis*, stomatal number is increased in newly emerging leaves treated by higher CO_2 concentrations or light intensity. Environmental cues can be sensed in mature leaves and then affect stomatal development in the developing leaves through long-distance signaling. One component, designated HIGH CARBON DIOXIDE (HIC) which encodes a putative 3-keto acyl coenzyme A synthase, has been identified to control stomatal number in response to elevated CO_2 (Gray et al., 2000). As understanding of stomatal development is largely advanced, it remains unclear how environmental signals are sensed and are subsequently incorporated into the developmental and patterning pathways to finely modulate stomatal number and distribution.

In recent years, much has been learned about the diversity of signal transduction mediated by plant G-proteins in *Arabidopsis* and rice (Perfus-Barbeoch et al., 2004). For many developmental processes, G-proteins play an important role in regulating cell proliferation (Ullah et al., 2001; 2003; Chen et al., 2003). Compared to animals, plants have a smaller number of heterotrimeric G-proteins. The *Arabidopsis* genome contains genes encoding only one canonical G-protein α-subunit (Gα), one β-subunit (Gβ), two γ-subunits (Gγ), one Regulator of G-protein Signaling (RGS), and a few putative G-protein-coupled receptors (GPCRs) (Jones and Assmann, 2004; Offermanns, 2003). The role of heterotrimeric G-proteins in plant cell division is contingent on cell types. For example, null alleles of *Arabidopsis* Gα subunit (*gpa1*) exhibit a reduced number of lateral root primordial, whereas null alleles of Gβ subunit (*agb1*) enhance cell division in roots and produces excessive lateral roots (Ullah et al., 2003). In addition, null alleles of *RGS1* or overexpression of a constitutively active GPA1 confer an increased cell division in the root apical meristem (Chen et al., 2003), indicating that the GTP-bound form of GPA1 plays a positive role in cell proliferation. In contrast, heterotrimeric complex acts as an attenuator of cell proliferation in the root apical meristem (Chen et al., 2006a, 2006b, 2006c). In animals, heterotrimeric G-proteins are also crucial for asymmetric cell division to generate cell diversity in addition to their role in cell proliferation (Gutkind, 1998). Here we provide a line of genetic evidence that plant G-protein signaling is involved in regulation of the frequency of asymmetrical cell divisions which is required for stomatal development in *Arabidopsis*. GPA1 and AGB1 modulate stomatal density in an opposite direction but have no effect on one-celled spacing. Deletion of GPA1 reduces stomatal density, whereas loss-of-function of AGB1 enhances stomatal density. The results support a proposition that plant G-proteins can accurately adjust cellular activities to maximally adapt environmental changes in multiple ways.

Materials and methods

Plant materials and growth conditions

Wild type plants used in this study were Columbia-0. All the mutants and transgenic lines used were in the Col-0 background. Mutants (*gpa1*, *rgs1*, *agb1*, *gpa1 agb1*) and transgenic lines (GPA^{QL}, AGB1ox) were as described by Chen et al. (2006). Seeds were surface-sterilized and grown in sterile culture on half strength Murashige and Skoog agar medium with 1% sucrose. Plates were incubated under light conditions of 70 μmol m^{-2} s^{-1} with 10-h-light/14-h-dark cycles and a constant relative humidity of 40% at 23 °C in the chamber. Cotyledons of 7-day-old seedlings were used in experiments, otherwise indicated specifically.

Confocal microscopy and stomatal statistics

For each genotype, stomatal numbers were recorded from 10 cotyledons. To visualize outlines of abaxial epidermal cells, cotyledons were immersed in 1 μM FM4-64 for 30 min, and mounted on slides with the cotyledon abaxial side facing up. Images were taken with a confocal laser-scanning microscope (LSM 510 META, ZEISS). According to the method of Claudia Kutter et al. (2007), the stomatal density, stomatal index, and the proportion of primary and higher-order stomatal complexes were quantified from confocal image of abaxial surface each cotyledon.

RT-PCR analysis of MUTE and SPCH

Total RNA was extracted from 7-day-old cotyledons using RNAqueous (Ambion) according to the manufacturer's instructions. After DNase treatment, 1 μg of total RNA was used for reverse transcription. Subsequently, 1 μL of reverse transcription reaction was used as template for PCR amplification. For RT-PCR, the primers for *FAMA* were (Forward, 5′-GAGCTCGAGCAACTCCTACAAT; Reverse 5′-GAAGTCGTTGTCGTTGTCATGT), for *TMM* were (Forward, 5′-TCCTTCACCTAGAGGGCAATAA; Reverse, 5′-ACGGTACTGGTCCTGTCAGACT), for *YDA* were (Forward, 5′-CACCATGAGATCACTGGACATT; Reverse 5′-GCCATGTTTTAATCCTTTCTGC), for ERECTA were (Forward, 5′-GATAATGTCAAAGACGGGGAAC; Reverse, 5′-GGAAAACTTTCTTCACCACACC), for MUTE were (Forward, 5′-CATCAAAAGGGGAGATCAAG; Reverse 5′-CAGAGATGATCTTTACGAGC), for *SPCH* were (Forward, 5′-AAAATCGGCTTTGGCTGATGTGAAG; Reverse, 5′-AGAAAGTGAGTACGTACTGC), and for *RBS* were (Forward, 5′-ATGGTGATGGCTGGTGCTTCTTCTTTGGA; Reverse, 5′-TTAGAGAGGAACGCTGTGCAAGACGACT). The PCR products were examined on a 1.2% agarose gel stained with ethidium bromide. The same RNA samples and primers were used for real-time PCR analysis. SYBR green was used as the intercalating dye. As an internal control, the RBS transcript was used to quantify the relative transcript level of each target gene. Quantitative RT-PCR analysis was performed using an Optican-2 real-time PCR machine (MJ Research). The thermal cycling conditions were as follows: 5 min in 96 °C, followed by 30 cycles of 95 °C for 15 s, 60 °C for 15 s, and 72 °C for 15 s. Relative levels of each transcript were calculated on the base of transcript levels of wild type. Three replicate biological experiments were conducted.

GUS staining

One-day-old *Arabidopsis* seedlings were incubated in GUS staining buffer (10 mM EDTA, 0.1% Triton x-100, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 100 μg/mL chloramphenicol, and 1 mg/mL 5-bromo-4-chloro-3-indolyl-β-glucuronic acid in 50 mM sodium phosphate buffer, pH 7.0) for 6 h at 37 °C. The seedlings were then cleared in 20% lactic acid and 20% glycerol and observed on an Olympus IX-70 microscope under Nomarski optics (magnification, ×180). The total number of stomata per cotyledon was quantified from GUS staining images. Counts were made of ten different cotyledons.

Drought tolerance and water loss

Seven-day-old seedlings grown on the 1/2 MS medium were air-drought-stressed through opening the cover of plates. The relative humidity decreased from 80% of inside of the plate to 40% of ambient. Picture was taken with Olympus IX-70 microscope after treatment for 45 min. For water loss assay, 50 cotyledons were detached from 7-day-old seedlings, and weighed immediately as fresh weight, then placed in controlled conditions and weighed at indicated time intervals. Water loss was measured and expressed as the percentage of initial fresh weight. In all of the drought tolerance and water loss experiments, seedlings or detached cotyledons were put under continuous 70 μmol m^{-2} s^{-1} fluorescent cool white light at 23 °C and 40% of relative humidity.

Results

GPA1 positively regulates stomatal development

It was reported that stomatal number increases in the hypocotyl epidermis overexpressing either the wild-type form or the constitutively

active form of the G-protein α subunit GPA1 (Okamoto et al., 2001). To explore how G-protein signaling regulates stomatal development, we examined the number of stomata in the single mutants, *gap1* and *rgs1*. Stomatal density, the number of stomata per unit area, was measured on the abaxial surface of cotyledons of 7-day-old seedlings grown on half MS media under white light (70 μmol m^{-2} s^{-1}). As shown in Fig. 1, stomatal density was significantly reduced in *gpa1* while enhanced in *rgs1*, which was detected to have an elevated steady-state pool of activated Gα (Chen et al. 2003). These data suggest a positive role of the active form of GPA1 in stomatal development. To confirm this hypothesis, we examined stomatal density in cotyledons of transgenic plants that overexpressed the constitutively active form of $GPA1^{QL}$. Our data showed that overexpressing $GPA1^{QL}$ led to an increase in stomatal density (Figs. 1A, B).

To exclude possibility that difference in stomatal density was caused by change in epidermal cell size, stomatal index (SI), which is defined as the ratio of the number of stomata to the number of epidermal cells plus stomata, was measured in the abaxial surface of cotyledons as described by Salisbury (1927). We found that the stomatal index was also significantly lower in *gpa1* (23.1%) than in the wild type (32.0%). Likewise, somatal index of the *rgs1* mutant and the GPA^{QL} transgenic plants increased (Fig. 1C). Together, the results suggest that the Gα subunit plays a positive role in regulating stomatal development in cotyledons.

AGB1 counteracts the action of GPA1 on stomatal development

Since G-protein signaling can be transmitted through the activated Gα, the Gβγ dimer, and/or the heterotrimeric complex, we then examined whether Gβ could influence stomatal development. In contrast to the Gα mutant, inactivation of the Gβ subunit (*agb1*) exhibited a clear increase in stomatal density by ~24.7% in the cotyledon abaxial surface compared with that of the wild type. To further confirm that the Gβγ is a negative regulator of stomatal development, stomatal density was measured in the transgenic lines overexpressing *AGB1*. Overexpression of *AGB1* (AGB1ox) reduced stomatal density on the abaxial epidermis of cotyledons (Figs. 2A, B). A similar result was observed if stomatal number was evaluated by stomatal index (Fig. 2C). Therefore, we concluded that AGB1 is a negative regulator of stomatal development. Since a phenotype which is opposite in *gpa1* and *agb1* mutants usually indicates that G-protein signaling is mediated by Gβγ in *Arabidopsis* (Pandey et al., 2008), we tested whether AGB1 is the only signaling branch to regulate stomatal development. Stomatal density and index were examined in cotyledons of the *gpa1 agb1* double mutant. Stomatal density and index were higher in *gpa1 agb1* than in the wild type but lower than in *agb1* (Figs. 2B, C), suggesting that the genetic relationship between GPA1 and AGB1 in stomatal density regulation can be intricate. They might function antagonistically in the same pathway or in different pathways for stomatal development.

G-proteins regulate the proportion of the primary and higher-order stomatal complexes in a fine way

As explained above, stomatal number is mainly dependent on the frequency of asymmetrical cell divisions. Generally, meristemoids are able to undergo up to 3 rounds of asymmetric divisions before differentiating into GMCs (Pillitteri et al., 2007). Each stoma with all its associated subsidiary cells is considered a discrete unit (a stomatal complex). Fig. 3A showed representative confocal microscopy images of individual primary, secondary and tertiary stomatal complexes. To dissect the modified process of stomatal development in G-protein mutants, we traced the cell lineage of individual stomatal complex. The number of the primary, secondary and tertiary stomatal complexes on the abaxial surface was analyzed with at least ten cotyledons for each genotype. Our results showed that loss-of-function of *GPA1* conferred a markedly increased number of the primary stomatal complex but lacked the secondary and tertiary stomatal complexes compared with the control. In contrast, the mutation in *AGB1* led to produce a higher proportion of the tertiary stomatal complex while a lower proportion of the primary and secondary stomatal complexes than the control

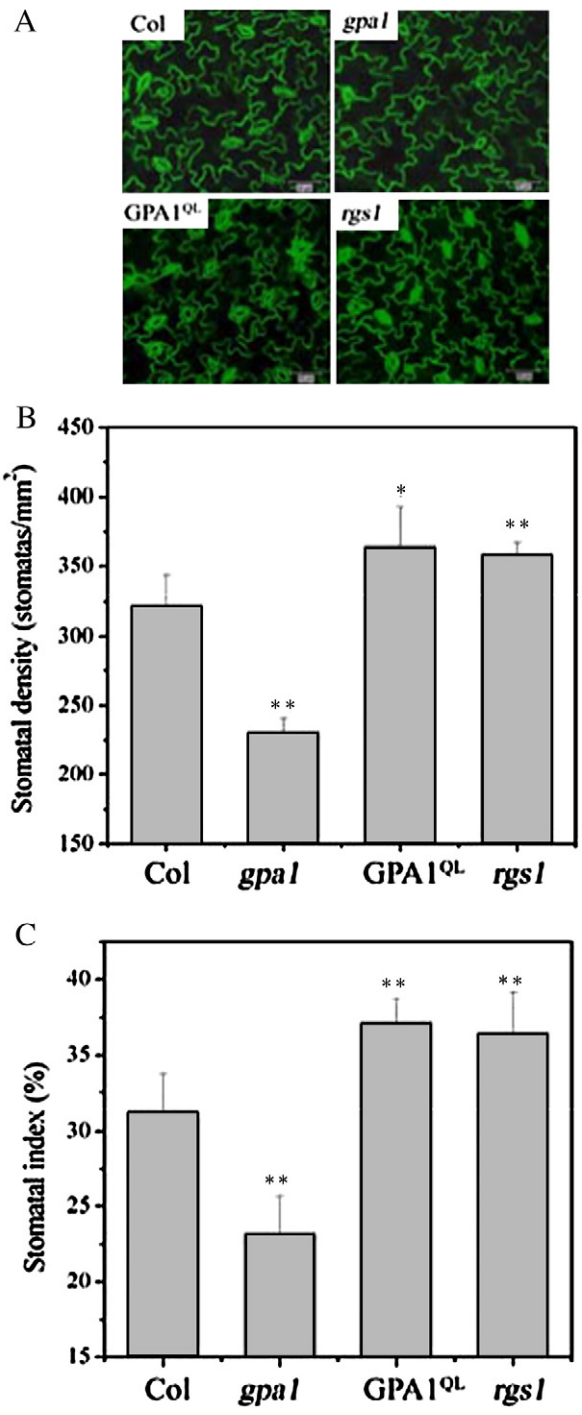

Fig. 1. GPA1 is a positive regulator of stomatal development. Seedlings were grown on half strength of MS media supplemented with 1% sucrose under light condition of 70 μmol m^{-2} s^{-1} and 10-h-light/14-h-dark cycles at 23 °C. (A) Confocal images of stomata on the abaxial epidermis of cotyledons from 7-day-old seedlings of the wild type (Col), *gpa1* and *rgs1* mutants, and the transgenic line expressing the constitutively active form of $GPA1^{QL}$. Cell outlines were visualized with FM4-64 staining. Bras = 50 μm in every picture. (B and C) Stomatal density and index calculated from panel A, respectively. The data present average values of ten individual cotyledons ± SD. * and ** denote significant differences between mutant and wild-type plants at the level of $P = 0.05$ and $P = 0.01$ by a *t* test, respectively.

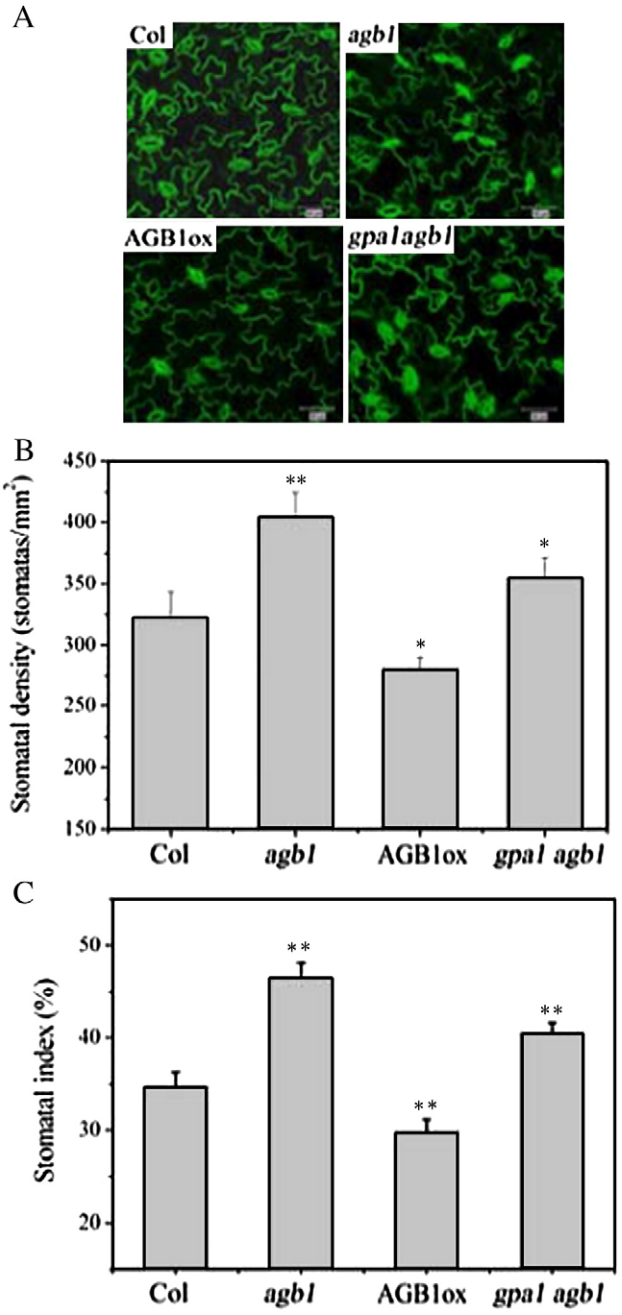

Fig. 2. AGB1 negatively modulates stomatal development in a GPA1-independent manner. Conditions of seedling growth and technique to determine stomatal number was the same as described in Fig. 1. (A) Confocal images of stomata on the abaxial epidermis of cotyledons from 7-day-old seedlings of the wild type (Col), *agb1* and *gpa1agb1* mutants, and the transgenic line (AGB1ox) overexpressing AGB1. Bars=50 μm in every picture. (B and C) stomatal density and index of Col, *agb1*, AGB1ox, *gpa1 agb1*. The data represent average values of ten individual cotyledons±SD. *, significant (P=0.05); **, highly significant (P=0.01).

(Fig. 3B). However, we did not observe that stomata were abnormally clustered or the one-cell spacing rule was affected in G-protein mutants. These results may reflect that G-proteins influence stomatal development mainly through modulating the frequency of amplifying divisions occurring in meristemoids.

Expression of SPCH and MUTE was up-regulated in agb1 but down-regulated in gpa1

To date, many genes that control the production and spacing of stomata have been identified in *Arabidopsis*. These genes include putative receptors, a processing protease, a MAP kinase cascade, and transcriptional factors that act at either the early stage or the later stage in stomatal development. In order to understand molecular mechanism underlying the effect of G-proteins on stomatal development, we examined the expression level of genes such as *ERECTA*, *YDA*, *TMM*, *FAMA*, *SPCH* and *MUTE* in cotyledons of 7-day-old seedlings by RT-PCR using gene specific primers. Interestingly, we detected transcriptional difference of *SPCH* and *MUTE*, but not *ERECTA*, *YDA*, *TMM* or *FAMA* among the wild type, *gpa1* and *agb1* (Fig. 4A). Transcript levels of *SPCH* and *MUTE* decreased in *gpa1* moderately while increased in *agb1*. This result was further confirmed by quantitative real-time PCR (Fig. 4B). The data showed that mRNA levels of *SPCH* and *MUTE* decreased by 48.3% and 55.2% in *gpa1* compared with that of the wild type, respectively. In contrast, mRNAs of *SPCH* and *MUTE* accumulated 60.2% and 33.7% more in *agb1* than in the wild type, respectively. It has been proposed that the number of stomata is mainly influenced by two factors: entry divisions of postprotodermal cells and later amplifying asymmetric divisions of meristemoids. SPCH is required for the first asymmetric entry division into the stomatal lineage (MacAlister et al., 2007; Pillitteri et al., 2007), while MUTE is a key gene determining the number of satellite meristemoids as well as stomatal differentiation from meristemoids to GMCs (Pillitteri et al., 2007). Thus, our gene expressing data were consistent with changes in stomatal density and patterning in G-protein mutants, suggesting a possible role of *SPCH* and *MUTE* in G-protein-mediated stomatal development.

The number of meristemoids is altered in gpa1 and agb1 cotyledons

Because *MUTE* is expressed strongly in a subset of meristemoids but weakly in GMCs and guard cells (Pillitteri et al., 2007), it is a suitable marker gene to identify meristemoids at the early stage of stomatal development. To further confirm whether alteration in the proportion of different stomatal complexes in G-protein mutants was associated with the number of meristemoids, we introduced the

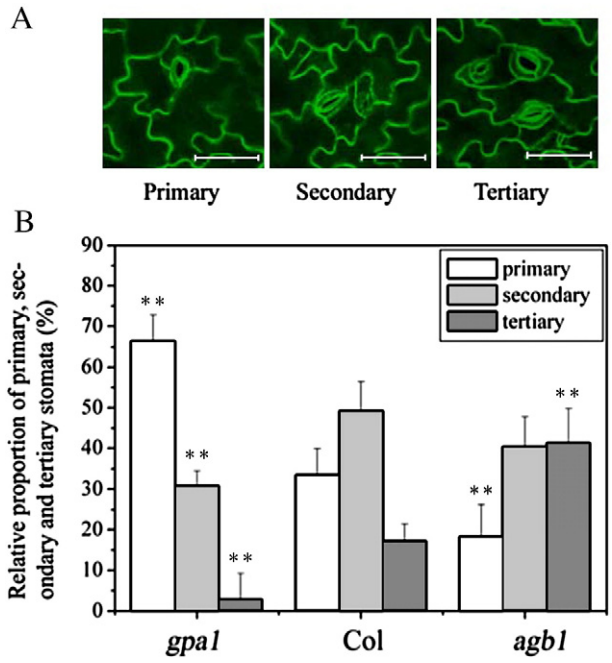

Fig. 3. Effect of GPA1 and AGB1 on the primary and higher-order stomatal complexes. (A) Representative confocal microscopy images of stomatal complexes on the abaxial surfaces of cotyledons. Bars=50 μm. (B) the proportion of primary, secondary, and tertiary stomatal complexes on the abaxial surfaces of the 7-day-old cotyledons of *gpa1*, Col and *agb1*. The proportion of stomatal complex types was compared between mutants and the wild type. *, significant (P=0.05); **, highly significant (P=0.01).

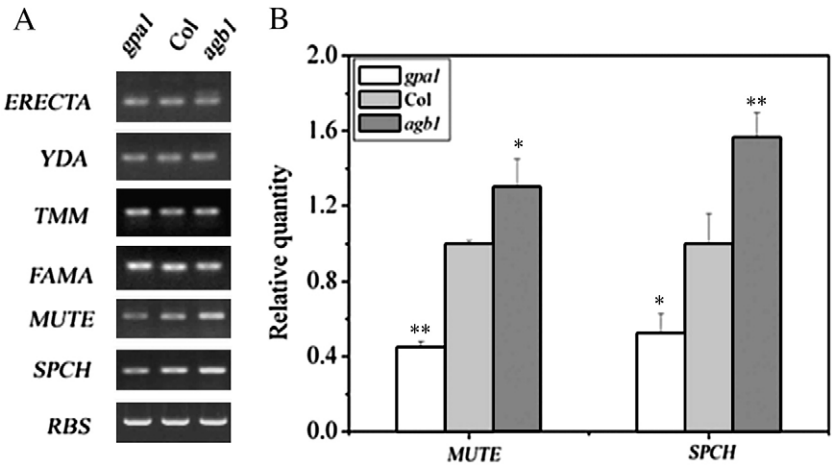

Fig. 4. Gene expression of key regulators for stomatal development in *gpa1* and *agb1*. Total RNA was abstracted from cotyledons of 7-day-old seedlings. (A) RT-PCR analysis of transcript levels of *ERECTA*, *YODA*, *TMM*, *FAMA*, *MUTE* and *SPCH* in the wild type, *gpa1* and *agb1* seedlings. The experiment was repeated three times, and a representative result of RT-PCR product was presented. *MUTE* and *SPCH* were up-regulated in *agb1* mutant but down-regulated in *gpa1* mutant compared with that in the wild type. (B) Transcriptional levels of *MUTE* and *SPCH* were subject to be analyzed by quantitative real-time RT-PCR. The transcriptional levels were first normalized to the expression of *rubisco small subunit* (*RBS*), and then the value for expression of each gene in the wild type was set to 1. Data are means ± SD of three independent experiments. *, significant (P=0.05); **, highly significant (P=0.01).

construct containing 1.9 kb of the *MUTE* promoter region driving the expression of β-glucuronidase (*MUTEpro:GUS*) into *gpa1*, *agb1* and the control plants. The activity of *MUTE* promoter was examined 24 h after seeds germination through GUS staining. As shown in Figs. 5A and B, the number of cells with GUS staining in a cotyledon was less in *gpa1* (49.5) while more in *agb1* (69.3) compared with the wild type (60.3), indicating that the number of meristemoids/somata is apparently altered in different genetic backgrounds. These results may indicate the action of G-proteins to the site of meristemoid regeneration in the course of stomatal development.

Water loss of cotyledons is reduced in gpa1 but enhanced in agb1

Transpiration water loss through stomata is a key determinant of drought tolerance (Xiong et al., 2002). To test if the G-protein-mediated stomatal development was associated with the rate of water loss, 7-day-old seedlings grown on half MS in covered Petri dishes were transferred to 40% of the relative humidity after covers were removed. Cotyledons of *agb1* and *gpa1 agb1* seedlings exhibited a more severe symptom of water loss than those of the wild type (Fig. 6A). However, under the same condition, *gpa1* cotyledons kept water turgor more strongly than wild-type ones (Fig. 6A). These results suggest that stomatal density regulated by G-proteins is proportional to the rate of water transpiration. In order to quantify differences in drought resistance among G-protein mutants and the wild type, transpiration rates of detached cotyledons were measured by their fresh weight changes over every 5 min under the controlled environment. The rate of water loss was significantly faster in *agb1* and *gpa1 agb1*, but slower in *gpa1* than the wild type (Fig. 6B). After 30 min, the relative water loss of *gpa1* and wild-type cotyledons was 63.7% and 78.9%, respectively. In contrast, as high as 87.4% and 88.5% water loss was detected for *agb1* and *gpa1 agb1* cotyledons (Fig. 6B). Taken together, these results suggest that G-protein-mediated stomatal development plays a role in controlling water transpiration rate of seedlings.

Discussion

Signaling through G-proteins is highly conserved in all eukaryotes. Despite the presence of a smaller number of G-protein complexes in higher plants, G-protein signaling pathways have been demonstrated to mediate various critical cellular processes. Here, we described another function of G-proteins that engaged in a fine regulation of stomatal development in *Arabidopsis* cotyledons. Because asymmetrical cell divisions are required for the initiation of the stomatal cell lineage, our findings suggest that G-proteins are involved in regulation of asymmetric divisions. Two G-protein subunits, GPA1 and AGB1, play opposing roles in this process, providing a new insight of G-protein-mediated cell divisions.

Antagonistic regulation of G-protein signaling via GPA1 and AGB1 on stomatal development

In this study, we found that the mutation of GPA1 led to decrease in stomatal density of cotyledons, suggesting that GPA1 is a positive factor adjusting stomatal density in response developmental and/or environmental cues. This phenotype can be attributable to either lack

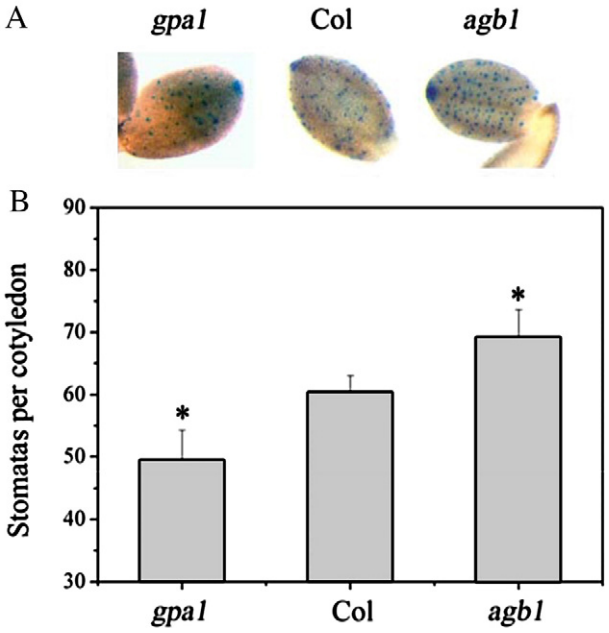

Fig. 5. The number of meristemoids analyzed by histochemical localization of *MUTEpro:GUS* expression 24 h after seed germination in cotyledons of wild-type, *gpa1* and *agb1* seedlings. (A) *MUTEpro:GUS* reporter expression in cotyledons. GUS staining is restricted to guard mother cells and developing guard cells. Bars = 10 μm. (B) the total number of guard cells and guard mother cells per cotyledon. The data represent average values of ten individual cotyledons ± SD. *, significant (P=0.05); **, highly significant (P=0.01).

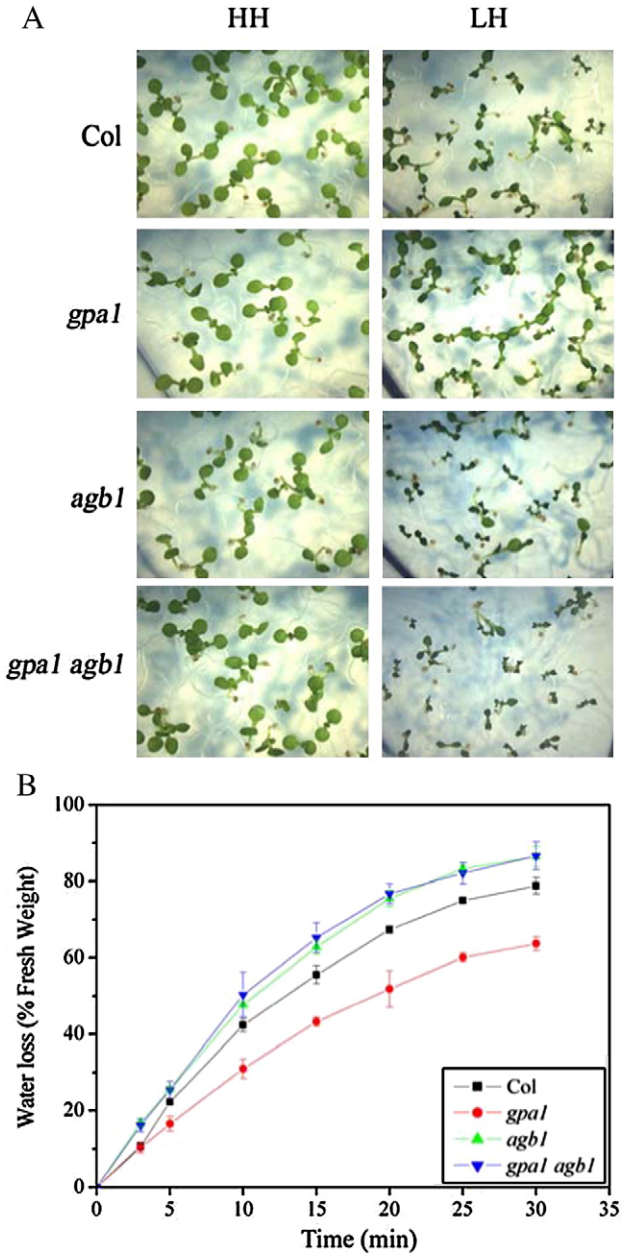

Fig. 6. Water loss of 7-day-old seedlings and detached cotyledons. (A) Phenotypic comparison of *gpa1*, *agb1*, *gpa1 agb1* and wild-type cotyledons in water loss. Seven-day-old seedlings grown in Petri dish were exposed to air with relative high humidity or relative low humidity after the cover was removed. Photos were taken 45 min after initiation of drought stress. HH, high humidity (80%); LH, low humidity (40%). (B) Water loss rates of detached cotyledons. Water loss was expressed as the percentage of initial fresh weight. Data are means ± SD of three independent experiments with 50 cotyledons per experiment.

of Gα subunit or permanent presence of free Gβγ dimmers. Further analyses of stomatal density in cotyledons of *rgs1* or transgenic plants that expressed the constitutively active form of GPA1^{QL} verify that the activated form of GPA1 is responsible for promotion of stomatal development. The data are consistent with previous reports showing that the number of stomata increased in hypocotyls of seedlings overexpressing GPA1^{QL} (Okamoto et al. 200), and that leaves over-expressing *RGS1* have lower stomatal density compared with the wild type (Chen et al. 2006). G-protein signaling through the GTP-bound form of GPA1 has been shown to confer altered sensitivities to D-glucose (Huang et al., 2006), cell proliferation in the root apical meristem (Chen et al. 2006) and the phytochrome A signaling pathway (Wei et al., 2008). Recent studies of G-protein complexes isolated from the plasma membrane of wild-type plants reveal that about 30% of total Gα is present in large macromolecular complexes of approximately 700 kD associated with Gβ, whereas the other Gα exists in free Gα (Wang et al., 2008). Considering that plant Gα has its high spontaneous nucleotide exchange coupled with a low GTPase activity, the free form of Gα is probably in the GTP-bound activated form which may directly signal to downstream effectors independently of Gβγ signaling. In addition, the activated form of Gα can also be generated from receptor-coupled dissociation of trimeric G-protein complexes.

On the other hand, our genetic data also revealed involvement of Gβγ signaling in stomatal development. In contrast to decrease in stomatal density in *gpa1*, loss-of-function of *AGB1* results in an obvious increase in stomatal density in cotyledons. Consistently, overexpression of *AGB1* led to reduced stomata density compared with the wild type. Thus, AGB1 may act as a negative regulator of stomatal formation. Since stomatal density is higher in *agb1* cotyledons than that in *rgs1* or GPA1^{QL}-overexpressing transgenic cotyledons, the possibility is low that the observed increase in stomatal density in *agb1* is resulted from the positive role of the active form of Gα. Thus, the antagonistic action between GPA1 and AGB1 in stomatal density reveals that both Gα and Gβγ can transduce signals to downstream effectors. This prediction is supported by stomatal density in the double *gpa1 agb1* mutant which exhibits the mean value of the single *gpa1* and *agb1* mutants. Such antagonistic effect of GPA1 and AGB1 has been observed in lateral root formation (Ullah et al., 2003; Chen et al., 2006). However, signaling via G-proteins is obviously different between stomatal density and lateral root formation. In the latter case, AGB1 acts downstream of GPA1 and both loss-of-function of *rgs1* and overexpression of a constitutively active form of GPA1^{QL} have no effect on lateral root formation. In contrast, two branch pathways mediated by GPA1 and AGB1 upon activation of G-proteins are contributable to stomatal development. In animals and yeast, antagonistic or cooperative regulation of signaling pathways by both the Gα and Gβγ subunits has been well documented (Jordan et al., 2000; Slessareva et al., 2006). Our data support the hypothesis that both the Gα- and Gβγ-subunits can simultaneously regulate the same developmental process in plants.

Effect of G-protein signaling on asymmetrical cell division

In animals, heterotrimeric G-proteins are crucial for asymmetric cell division, which relies on the spindle being asymmetrically positioned during mitosis (Gönczy, 2002). Although subcellular localization of GPA1 and AGB1 is found in the newly formed cell plane (Chen et al., 2006), implying a possible role of G-proteins in the position of cell plane development, no experiments have been conducted to examine whether G-proteins are involved in plant asymmetrical cell divisions. Stomatal formation is a suitable system to address this question since asymmetrical divisions are required for the generation of meristemoids. It seems that plants own two signaling pathways in control of stomatal development. One is well characterized and composed of a ligand, several receptor-like kinases, and a MAPK cascade. This pathway is likely to respond developmental cues as well as plays an important role in maintaining the one-cell-spacing role. The other one, which is yet to be elucidated probably modulates stomatal density without disrupting the one-cell-spacing role under various environmental conditions. In this study, we found that G-protein-related mutants only exhibit the change in stomatal density, but do not disrupt the rule of one-cell spacing in cotyledons. This finding aids in addressing whether G-protein signaling is involved in regulation of stomatal density under environmental stresses. Meanwhile, gibberellins and ethylene have been shown to promote stomatal formation in hypocotyls (Saibo et al., 2003; Serna and Fenoll, 1997; Gao et al. 2008), it is possible that G-protein-regulated stomatal

development is associated with phytohormone signaling pathways in *Arabidopsis*.

Detailed examination of the ratio of different stomatal complexes indicated that Gα might promote amplification of satellite meristemoids, whereas Gβ acts oppositely to Gα. This hypothesis is generally in agreement with the well-established role of the G-protein signaling pathway in the modulation of cell division (Ullah et al., 2001 and 2003; Chen et al., 2003) and the strongly meristem-expressed pattern of *GPA1*, *AGB1* and *RGS1* (Chen et al., 2006; Anderson and Botella, 2007; Chen et al., 2006). For example, GPA1 mutation often leads to a reduced number of cells in many tissues such as hypocotyls and leaves, whereas AGB1 mutation results in production of more cells in lateral root formation (Ullah et al., 2003). The interesting is that GPA1, AGB1, and RGS1 can all function in regulation of stomatal density. In *Arabidopsis*, the entry division to initiate the stomatal cell lineage is highly associated with the positive regulator SPCH. Loss-of-function of SPCH has no ability to make any stomata (MacAlister et al., 2007). Another important factor regulating asymmetric divisions is MUTE, a negative regulator, by terminating asymmetric divisions and promoting differentiation of stomata (Pillitteri et al., 2007). Expression levels of SPCH and MULE are moderately down-regulated in *gpa1* but up-regulated in *agb1* at day 7 after seed germination. We reason that the G-proteins may regulate the frequency of entry and/or amplifying divisions to alter stomatal density in the epidermis of *Arabidopsis*.

A role of G-proteins in fine regulation of water use efficiency

Due to the sessile nature, plants have evolved various highly-ordered sensing systems to dynamically monitor and maximally utilize natural resources through both developmental and physiological processes. Stomatal pores are known to play an important role in balancing CO_2 intake with water loss through regulation of stomatal movement and stomatal density. It is well documented that both stomatal development and movement can be regulated by plant hormones, such as GA, ethylene and ABA, and environmental factors including light intensity and CO_2 concentrations (Casson and Gray, 2008). Up to date, only the role of the G-protein signaling pathway in stomatal movement has been intensively studied in the presence of ABA. Genetic and physiological results reveal that the Gα subunit is required for the inhibition of stomatal opening in ABA-mediated stomatal movement (Wang et al., 2001). Consequently, the *gpa1* mutant has a greater rate of water loss than wild-type plants and is prone to be suffered from drought stress. It is interesting that the deletion of GPA1 leads to a reduction in the number of stomata, which can compensate for excessive water loss due to insensitivity of the stomata to ABA signaling. Actually, a similar outcome of GPA1 has been also observed in the epidermal layer (data not shown) where the cell number is significantly reduced while the cell size becomes larger in *gpa1* compared with the wild type (Ullah et al., 2001). Since amount of water loss from leaves is related to both stomatal density and movement, the importance of these two factors in drought resistance may vary according to growth conditions and developmental stages. Under our experimental conditions water loss in *gpa1* leaves is lower than in wild-type leaves, indicating that stomatal density play a more important role in regulation of water transpiration compared with stomatal movement. This result is consistent with the previous report showing that decrease in stomatal density conferred to higher water use efficiency of *Arabidopsis* leaves (Chen et al., 2006; Josette et al., 2005; Yu et al., 2008). However, the compensating effect observed in *gpa1* is not observed in *agb1*. Recently, inhibition of inward K^+ currents and stomatal opening by ABA is also shown to be impaired in *agb1* (Fan et al., 2008). This is consistent with our data that *agb1* is more sensitive to drought stress than *gpa1*. The synergistic regulation of AGB1 in stomatal density and movement might be important in some special surroundings such as high humidity and low levels of carbon dioxide. In summary, our findings reveal another side of G-proteins in fine regulation of inevitable water loss when plants take up the CO_2 required for photosynthesis through stomata.

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